DNA double-strand break repair is involved in desiccation resistance of *Sinorhizobium meliloti*, but is not essential for its symbiotic interaction with *Medicago truncatula*

Pierre Dupuy, Benjamin Gourion, Laurent Sauviac and Claude Bruand*

**Abstract**

The soil bacterium *Sinorhizobium meliloti*, a nitrogen-fixing symbiont of legume plants, is exposed to numerous stress conditions in nature, some of which cause the formation of harmful DNA double-strand breaks (DSBs). In particular, the reactive oxygen species (ROS) and the reactive nitrogen species (RNS) produced during symbiosis, and the desiccation occurring in dry soils, are conditions which induce DSBs. Two major systems of DSB repair are known in *S. meliloti*: homologous recombination (HR) and non-homologous end-joining (NHEJ). However, their role in the resistance to ROS, RNS and desiccation has never been examined in this bacterial species, and the importance of DSB repair in the symbiotic interaction has not been properly evaluated. Here, we constructed *S. meliloti* strains deficient in HR (by deleting the recA gene) or in NHEJ (by deleting the four ku genes) or both. Interestingly, we observed that ku and/or recA genes are involved in *S. meliloti* resistance to ROS and RNS. Nevertheless, an *S. meliloti* strain deficient in both HR and NHEJ was not altered in its ability to establish and maintain an efficient nitrogen-fixing symbiosis with *Medicago truncatula*, showing that rhizobial DSB repair is not essential for this process. This result suggests either that DSB formation in *S. meliloti* is efficiently prevented during symbiosis or that DSBs are not detrimental for symbiosis efficiency. In contrast, we found for the first time that both recA and ku genes are involved in *S. meliloti* resistance to desiccation, suggesting that DSB repair could be important for rhizobium persistence in the soil.

**INTRODUCTION**

Double-strand DNA breaks (DSBs) are considered the most deleterious DNA damages in living cells, as a single DSB can lead to cell death if not repaired [1]. In bacteria, two predominant DSB repair pathways have been described: homologous recombination (HR) and non-homologous end-joining (NHEJ). HR is a universal mechanism that has been largely characterized in recent decades, which allows DSB repair by a process based on exchange of genetic material between two homologous DNA sequences [2]. RecA is the key factor of HR, promoting single-strand DNA annealing and exchange during the initial synaptic steps of HR. RecA also contributes to the repair of other DNA damage through a pathway called the SOS response [3]. Accordingly, bacterial recA mutants are sensitive to a wide range of DNA damaging agents, which may or may not lead to the formation of DSBs [4]. On the other hand, NHEJ was discovered recently in bacteria, where it is present in only ~20% of species [5–7]. Bacterial NHEJ has been characterized in a limited number of species, such as *Bacillus subtilis* and *Mycobacterium* sp. In the NHEJ pathway, the Ku protein binds DNA ends and then recruits a multifunctional enzyme called LigD, which processes and covalently joins the DNA ends [8, 9]. In *B. subtilis* and *Mycobacterium*, Ku was shown to be important for resistance to several DSB-causing agents, including ionizing radiations (IRs) [7, 10]. HR is considered the main mechanism of DSB repair in bacteria, mainly active in exponentially growing cells when the genome is present in multiple copies, to satisfy the need for homologous DNA sequences. However, HR is considered inefficient in the stationary phase because of the reduced genome copy number due to inactive DNA replication, and NHEJ then becomes a major pathway [10, 11]. Thus, NHEJ has been shown to be important for the survival of bacteria submitted to lengthy non-replicating states of starvation, like *Mycobacterium* when internalized into macrophages or *B. subtilis* during sporulation [12, 13].
Sinorhizobium meliloti is a soil bacterium belonging to the alpha subclass of Proteobacteria, with active HR and NHEJ mechanisms, as suggested by independent phenotypic analyses of recA and ku mutants [14–16]. S. meliloti is able to establish a symbiotic relationship with legume plants, including Medicago spp., characterized by the formation of new plant root organs called nodules in which bacteria multiply and differentiate into bacteroids able to reduce atmospheric nitrogen to ammonium, directly usable by the plant [17]. S. meliloti is exposed to various stresses in nature [18], including DSB-causing conditions. For instance, desiccation is a well-known cause of DSB formation frequently encountered by free-living soil bacteria [10, 19]. However, whether HR and/or NHEJ are involved in S. meliloti resistance to desiccation is currently unknown. During the rhizobium–legume interaction, nitric oxide (NO) and hydrogen peroxide (H₂O₂) are present in root hairs and nodule tissues [20–23]. These reactive nitrogen species (RNS) and reactive oxygen species (ROS), involved in various steps of the symbiotic relationship, have been otherwise described to induce DSBs [24, 25]. However, whether DSB repair is important for S. meliloti to establish and maintain an efficient interaction with the plant is actually not known. S. meliloti recA and ku mutants were previously independently shown not to be affected in regard to their symbiotic efficiency with alfalfa (Medicago sativa) [14–16], suggesting that HR and NHEJ are not required individually. However, the possibility of a functional redundancy of the two repair systems has never been explored.

Here, we generated an S. meliloti strain deficient in both HR and NHEJ. Although this mutant presented clear defects in DSB repair under free-living conditions, it was fully efficient in symbiosis with Medicago truncatula, and the survival of mutant bacteria in nodules was equivalent to that of the WT. In contrast, deficiencies in HR and/or NHEJ strongly affected the ability of S. meliloti to survive desiccation. Together, these results indicate that DSB repair in S. meliloti is not essential for symbiosis per se, but may be required for bacterial survival in the soil.

**METHODS**

**Bacterial strains and growth conditions**

The strains used in this study are listed in Table 1. Escherichia coli strains were grown in Luria–Bertani (LB) medium at 37°C. S. meliloti strains were grown at 28°C, in LB medium supplemented with 2.5 mM CaCl₂ and 2.5 mM MgCl₂ (LBMC; used for strain constructions and precultures), in TY medium supplemented with 6 mM CaCl₂ (TYC), in yeast mannitol medium (YM; 0.5 g l⁻¹ K₂HPO₄, 0.2 g l⁻¹ MgSO₄, 0.1 g l⁻¹ NaCl, 0.5 g l⁻¹ yeast extract and 10 g l⁻¹ mannitol; pH=6.8) or in Vincent minimal medium (VMM; 7.35 mM KH₂PO₄, 5.74 mM K₂HPO₄, 1 mM MgSO₄·7H₂O, 456 µM CaCl₂, 35 µM FeCl₃, 4 µM biotin, 48.5 µM H₂BO₃, 10 µM MnSO₄, 1 µM ZnSO₄, 0.5 µM CuSO₄, 0.27 µM CoCl₂, 0.5 µM NaMoO₄, 10 mM sodium succinate and 18.7 mM NH₄Cl; pH=7). Antibiotics were added at the following final concentrations: 100 µg ml⁻¹ streptomycin (Sm), 40 µg ml⁻¹ gentamicin (Gm), 10 µg ml⁻¹ tetracyclin (Te), 40 µg ml⁻¹ spectinomycin (Spec) or 50 µg ml⁻¹ carbenicillin (Cb). To perform growth curves, overnight precultures grown in 5 ml LBMC medium supplemented with Sm were diluted to an optical density at 600 nm (OD₆₀₀) of 0.05 in 10 ml TYC supplemented with Sm, and were grown for 8 h. Cells were again diluted in fresh TYC (OD₆₀₀=0.001) supplemented with Sm, and growth was measured by monitoring OD₆₀₀ every 2 h from OD₆₀₀=0.1. To evaluate cell viability, bacterial cultures in TYC supplemented with Sm were grown to exponential (OD₆₀₀=0.3–0.6) or stationary (24 h) phase and were plated on LBMC supplemented with Sm after serial dilution.

**Strain and plasmid constructions**

All plasmid constructions were performed in E. coli DH5α. The absence of mutations in all constructs was checked by DNA sequencing. Gene deletions were performed using pJQ200mp19 derivatives containing −350 to 450 bp regions flanking the gene to be deleted: recA (Smc00760), ku1 (Sma0426), ku2 (Smb20686) and ku3 ku4 (Smb21406–Smb21407). ORF-flanking DNA fragments were amplified by PCR using S. meliloti GMI11495 genomic DNA as template and the oligonucleotides listed in Table S1 (available in the online Supplementary Material) as primers, and these were individually cloned into pGEM-T. These regions were then subsequently juxtaposed as Sall–BamHI and BamHI–SacI fragments into pJQ200mp19 digested with Sall–SacI.

Plasmids were introduced in S. meliloti by electrottransformation as previously described [26]. For the construction of deletion mutants, single-crossover genomic integration of the corresponding pJQ200mp19 derivatives was generated by selecting for Gm resistance. The resulting strains were then propagated in the absence of antibiotic, and cells having lost the plasmid by a second recombination event were selected by plating on LBMC supplemented with 5% sucrose (Suc). Suc₅ GM₃ colonies were screened by PCR analysis using OCB1339–OCB1340, OCB1321–OCB1322, OCB1323–OCB1324 or OCB1325–OCB1328 as primers for deletion of recA, ku1, ku2 or ku3 ku4, respectively. The druple ku mutant was constructed by successive deletions of ku2, ku3 ku4 and ku1. The ΔrecA Δku1234 strain was constructed by deletion of recA from the Δku1234 strain.

For the construction of plasmid pDP125, used for measuring HR frequencies, two −500 bp regions of the chromosomal gene rhaS flanking a Spec₅ gene were juxtaposed in plasmid pJQ200mp19 (construction details available on request).

**UV sensitivity assay**

To test UV sensitivity, bacterial cultures in TYC supplemented with Sm were grown to exponential (OD₆₀₀=0.3–0.6) or stationary (24 h) phase. Cultures were diluted at an OD₆₀₀ of 0.1 and serial dilutions (10 µl) were spotted on LBMC plates supplemented with Sm. The plates were...
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
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<tr>
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<tr>
<td>GM11495</td>
<td>WT strain (Sm&lt;sup&gt;B&lt;/sup&gt;), Rn2011 background</td>
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Plasmids

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<td>Promega</td>
</tr>
<tr>
<td>pJQ200mp19</td>
<td>Gene replacement vector (Gm&lt;sup&gt;B&lt;/sup&gt;)</td>
<td>[49]</td>
</tr>
<tr>
<td>pHIP45-T</td>
<td>Vector (Spec&lt;sup&gt;B&lt;/sup&gt;)</td>
<td>[50]</td>
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<tr>
<td>pDP90</td>
<td>pBBRMC53 derivative (Tet&lt;sup&gt;B&lt;/sup&gt;)</td>
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<tr>
<td>pDP125</td>
<td>pJQ200mp19 derivative for rhaS integration (Gm&lt;sup&gt;B&lt;/sup&gt;, Spec&lt;sup&gt;B&lt;/sup&gt;)</td>
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<td>pDP52</td>
<td>pJQ200mp19 derivative for recA deletion</td>
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submitted to UV radiation (wavelength=254 nm) at doses of 0, 15, 30 or 100 J m<sup>-2</sup> using a Stratalinker 2400 UV Crosslinker (Stratagene) and were then incubated at 28°C for 72 h.

**Recombination assay**

A total of 100 ng of the suicide plasmid pDP125 (Gm<sup>B</sup>) prepared from E. coli and 0.5 ng of the replicative plasmid pDP90 (Tet<sup>B</sup>) prepared from S. meliloti were simultaneously electrotransformed in the indicated S. meliloti strains, and transformants were selected on LBMC plates supplemented with either Gm or Tet. The frequency of HR (i.e. integration of pDP125 at the chromosomal rhaS locus) was calculated as the ratio of numbers of Gm<sup>B</sup> and Tet<sup>B</sup> transformants obtained from equivalent amounts of DNA.

**H<sub>2</sub>O<sub>2</sub>, peroxynitrite and NO sensitivity assays**

For the H<sub>2</sub>O<sub>2</sub> disc diffusion assay, bacteria were grown in TYC supplemented with Sm to exponential phase, diluted at an OD<sub>600</sub> of 0.1 and plated on LBMC plates. A filter disc was put on the plate and spotted with 5 µl of 1 M H<sub>2</sub>O<sub>2</sub> (Sigma H-0904). The diameter of the growth inhibition area was measured after incubation at 28°C for 72 h.

For H<sub>2</sub>O<sub>2</sub> or peroxynitrite liquid culture assays, bacteria were grown in TYC medium supplemented with Sm to exponential (OD<sub>600</sub>=0.3–0.6) or stationary (24 h) phase. Cultures were diluted at an OD<sub>600</sub> of 0.3 and subjected to 0 or 30 mM H<sub>2</sub>O<sub>2</sub> for 30 min or to 0 or 1 mM peroxynitrite (Calbiochem 516620 or Interchim 81565) for 2 h. Before peroxynitrite addition, bacteria were washed and resuspended in phosphate buffer (pH=8). After treatment, cultures were washed with TYC to remove H<sub>2</sub>O<sub>2</sub> or peroxynitrite and bacteria were serially diluted and plated (10 µl) on LBMC supplemented with Sm. Survival was calculated as the number of c.f.u. ml<sup>-1</sup> divided by the number of c.f.u. ml<sup>-1</sup> obtained with bacteria that did not undergo H<sub>2</sub>O<sub>2</sub> or peroxynitrite treatment.

For NO sensitivity assays, bacteria were grown in VMM supplemented with Sm to exponential phase and diluted at an OD<sub>600</sub> of 0.15. The NO donor Spermine NONOate (SpNN) (Cayman Chemical CAY-82150-M100) prepared in 0.01 M NaOH was added to cultures at a final concentration of 0, 400 or 800 µM. Cultures were then distributed in a flat-bottomed, 48-well plate (800 µl per well) and incubated at 28°C in a Fluostar Omega plate reader (BMG Labtech) in which OD<sub>600</sub> was measured every 10 min during 48 h.

**IR sensitivity assay**

Bacteria were grown in VMM medium supplemented with Sm to exponential (OD<sub>600</sub>=0.3–0.6) or stationary (24 h) phase. Cultures were washed and diluted at an OD<sub>600</sub> of 0.3 in 0.85 % NaCl and were aliquoted in 1.5 ml Eppendorf tubes. Bacteria were irradiated in a gamma irradiator (Bio-beam 8000) at doses of 0, 100 or 200 Gy (exposure times of 0, 30 and 60 min, respectively). Finally, serial dilutions were spotted (10 µl) on LBMC plates supplemented with Sm and incubated for 72 h at 28°C. Survival was calculated as the number of c.f.u. divided by the total number of c.f.u. obtained with bacteria that did not undergo IR treatment.

**Desiccation sensitivity assay**

Desiccation sensitivity was assessed using a method adapted from Humann et al. [27]. Briefly, YM agar was aliquoted (50 µl) into wells of a flat-bottomed, 96-well microtitre plate and allowed to solidify. S. meliloti strains were cultured to stationary phase (48 h) in TYC and 5 µl was dispensed into the wells (one strain per plate row). The plate was then sealed with an adhesive gas-permeable seal (Thermo Scientific), covered with the plate lid and incubated at 28°C. After two days, the plate lid was removed and a time zero colony count was performed. For this, the adhesive seal was removed from the first column and bacteria and agar plugs were resuspended in 200 µl of phosphate buffer, serially diluted and spotted (10 µl) on LBMC plates supplemented with Sm that were then incubated at 28°C for 72 h. The adhesive seal was folded back down on the first column and the plate was returned at 28°C. Every week, additional samples were rehydrated, resuspended and plated as described above.

**Plant assays**

*Medicago truncatula* Jemalong A17 was used, and plant inoculations were performed as described in Berrabah et al. [28] with minor changes. After germination, seeds were transferred to 12 cm square Petri dishes containing Fahraeus medium [29] covered with filter paper (paper support for seed germination and plant growth in pouches, Mega International) and incubated at 25°C. For measurement of nodule formation and symbiosis efficiency, a total of 48 plants were inoculated with each strain and assessed as previously described.
for each bacterial genotype were tested, in two independent experiments.

To enumerate surviving root nodule bacteria, nodules were collected from plants at 4 or 6 weeks post-inoculation using sterile tweezers and placed individually in a 2 ml Eppendorf tube. Nodules were scrambled using a ceramic ball with sand and the ground product was diluted in 1 ml of TY. Serial dilutions were spotted on TYC and plates were incubated for 72 h at 28°C. The numbers shown are the mean and SE of the results obtained from a total of 34 nodules for each bacterial genotype, collected in two independent experiments.

For measurement of nitrogen fixation efficiency using the acetylene reduction assay, each nodulated plant (4 weeks post-inoculation) was transferred to a 50 ml test tube sealed with a rubber stopper. One millilitre of air was removed from the tube using a syringe and replaced with 1 ml of acetone. The surface of the ethylene gas chromatograph (Agilent 7820A). The surface of the ethylene peak was then used to assess nitrogenase activity. The results shown [expressed in arbitrary units (AU)] are the mean and SEM of the results obtained from 10 plants for each bacterial genotype.

RESULTS
Characterization of *S. meliloti* recA and ku mutant strains

HR and NHEJ were inactivated by deletion of the recA and ku genes, respectively. As the *S. meliloti* genome contains four ku genes [15], we constructed the Δku1234 quadruple mutant to remove any possible trace of NHEJ activity.

![Graphs showing growth curves and plating efficiencies of different bacterial strains](image)

The constructed strains were then further characterized and assessed for their capacity to repair DNA damage. In rich liquid medium (TYC), the ΔrecA mutant strain displayed a slightly slower growth than the WT strain (doubling time 160 vs 140 min), while growth of the Δku1234 strain was indistinguishable from that of the WT (Fig. 1a). The size of the colonies obtained on rich solid medium (LBMC) was consistent with these phenotypes, with ΔrecA colonies slightly smaller than WT and Δku1234 colonies (see Fig. 2 and following). The recA mutant displayed a lower viability than the WT and Δku1234 strains, as shown by its threefold lower plating efficiency (Fig. 1b). This lower viability was observed with actively growing bacteria only, as plating efficiencies of stationary phase recA and WT bacteria were not significantly different from each other.

We tested the ability of the constructed strains to perform HR by measuring their capacity to integrate a non-replicative plasmid in the genome by recombination within a homologous chromosomal region. The ΔrecA strain was impaired in its capacity to perform HR, whereas the deletion of *ku1234* did not impact plasmid integration efficiency (Fig. 1c).

We measured the sensitivity of the constructed strains to UV radiation, a general test generally assumed to cause several types of DNA damage but only rare DSBs. At 30 J m⁻², while WT and Δku1234 survival was not reduced in comparison to unexposed cells, the ΔrecA mutant survival was reduced by 10³- to 10⁴-fold (fold) (Fig. 2). Interestingly, we noticed that bacteria in stationary phase were tenfold more UV resistant than bacteria in exponential phase, but again, only recA deletion increased the UV sensitivity of bacteria.

The combination of recA and ku mutations did not exacerbate the observed recA phenotypes, since the ΔrecA Δku1234 strain...
was indistinguishable from the ΔrecA strain when tested for growth rate, viability and UV sensitivity (Figs 1 and 2). Together, these data confirm that RecA is involved in HR and repair of DNA damage occurring spontaneously or upon UV irradiation in S. meliloti, whereas Ku proteins, thought to be involved only in NHEJ, are not [14, 16].

Finally, to show that HR and NHEJ do contribute to DSB repair in S. meliloti, we exposed WT, ΔrecA, Δku1234 and ΔrecA Δku1234 strains to gamma Irrs, known to induce several DNA injuries, including DSBs. In log phase (Fig. 3a), recA mutant bacteria displayed a higher IR sensitivity than WT bacteria. The ku1234 deletions did not affect IR sensitivity, as previously described [15], not even in combination with the recA mutation, suggesting that NHEJ is not involved in DSB repair in growing bacteria. In stationary phase (Fig. 3b), WT cells were more resistant to IR than in exponential phase. As in exponential phase, the ΔrecA mutant displayed a higher IR sensitivity than the WT strain. Interestingly, the Δku1234 mutant showed a similar phenotype, as previously described [15], and the ΔrecA Δku1234 strain was even more sensitive. These results show that both RecA and Ku proteins contribute to S. meliloti resistance against IR in stationary phase.

Involvement of RecA and Ku proteins in S. meliloti resistance to ROS and RNS

To investigate whether RecA or Ku proteins could play a role in S. meliloti survival against ROS and RNS, known to produce DSBs, we submitted bacterial cultures WT, ΔrecA, Δku1234 and ΔrecA Δku1234 to such compounds.

NO sensitivity was assessed by looking at the growth arrest of exponentially growing bacterial cultures upon addition of the NO donor Spermine NONOate (SpNN). As previously described [30], the addition of SpNN to S. meliloti cultures led to growth arrest whose length was dependent on the SpNN concentration (Fig. 4). All four strains were indistinguishable at SpNN concentrations <400 µM, but at higher SpNN concentrations ΔrecA strains recovered more slowly than the WT strain, whereas the Δku1234 strain did not differ from the WT. This observation suggests that RecA is involved in the repair of NO-induced DNA damage in S. meliloti. Since this assay can test only growing bacteria, we could not evaluate the NO resistance of stationary phase bacteria. To test the effect of the growth phase, we submitted either exponentially growing or stationary phase bacteria to peroxynitrite (ONOO⁻), a compound naturally produced in vivo by reaction of NO with superoxide, and which is known to induce a wide range of damage including DNA DSBs [31]. In exponential growth condition, ΔrecA survival was decreased ten fold in the presence of 1 mM ONOO⁻ as compared to the WT, whereas ku1234 deletion did not impact S. meliloti resistance, even in the recA context (Fig. 5a). In stationary phase, bacteria were more resistant to peroxynitrite, as no loss of survival was observed for any strain at 1 mM peroxynitrite (Fig. 5b). However, higher peroxynitrite concentrations led to high cell mortality, and we were not able to detect any significant difference between the various strains. In conclusion, we showed that RecA is involved in the repair of DNA damage induced by NO or its derivative, peroxynitrite.

Sensitivity to H₂O₂ was measured either using a disc diffusion assay (Fig. 6a) or directly in liquid culture (Fig. 6b). Growth inhibition halos of WT and Δku1234 strains were similar (diameter=25 mm), whereas they were wider for ΔrecA and ΔrecA Δku1234 strains (diameter=32 mm). This shows that RecA is involved in S. meliloti H₂O₂ resistance. To test the effect of the growth phase, we submitted either exponentially growing or stationary phase bacteria to H₂O₂ (30 mM for 30 min). In log phase (Fig. 6b), while the ku1234 deletions did not impact H₂O₂ resistance, the ΔrecA strains were ten fold less resistant than the WT, suggesting that HR (but not NHEJ) participates in H₂O₂ resistance in this culture condition. In stationary phase, whereas the recA and ku1234 deletions did not significantly impact cell sensitivity, the ΔrecA Δku1234 mutant was more sensitive than the WT strain. These results therefore suggest an involvement of both RecA and Ku proteins in the repair of DNA damage caused by H₂O₂.

S. meliloti RecA and Ku proteins are not required for symbiosis with M. truncatula but contribute to bacterial resistance against desiccation

The above results suggested that S. meliloti Ku and/or RecA proteins might be involved in the repair of DNA damage caused by ROS or RNS during symbiosis with legumes. Although RecA and Ku proteins were independently shown not to be involved in symbiosis with alfalfa (M. sativa), we repeated the experiment with mutant bacteria affected in both systems, and using the model legume M. truncatula. The ΔrecA Δku1234 mutant strain displayed a symbiotic phenotype indistinguishable from that of the WT strain, with similar
number and aspect of root nodules and identical growth of
the plants over 6 weeks (data not shown). Equivalent nitrogen
fixation efficiencies were also measured by acetylene reduction
assay (376±81 AU for the WT vs 367±51 AU for the ∆recA
Δku1234 strain). Equivalent numbers of c.f.u. were recovered
from WT or ∆recA Δku1234-induced nodules collected 6
weeks post-inoculation (1.6±0.2×10⁶ c.f.u. node⁻¹ for the
WT vs 9.5±1.3×10⁵ c.f.u. node⁻¹ for the ∆recA Δku1234
strain). Together, these results indicate that RecA and Ku pro-
teins are not required for nodule formation, bacteroid differ-
etiation or bacterial survival in nodules. Therefore, we
conclude that S. meliloti DSB repair is not essential for efficient
symbiosis with M. truncatula.

Desiccation is a major stress encountered in natura by soil
bacteria, including rhizobia [19], and is known to cause a
wide range of cell damage including DSBs [32, 33]. While
various systems have been identified which allow rhizobia
to resist desiccation [19, 27], nothing is known about the
involvement of DSB repair. To address this question, we
measured the desiccation sensitivity of strains WT, ∆recA,
Δku1234 and ∆recA Δku1234 (Fig. 7). S. meliloti was
affected by desiccation since WT survival was reduced by
−10³-fold after 21 days of desiccation. The persistence
of strains ∆recA and ∆recA Δku1234 was further reduced by
10⁻²- and 10⁻³-fold, respectively. Whereas the ku1234 deletion
alone did not significantly affect resistance after 21 days, the
survival of the Δku1234 strain survival was ten fold lower
than that of the WT strain after 40 days of desiccation.
These results therefore show that both RecA and Ku pro-
teins are involved in desiccation resistance in S. meliloti.

**DISCUSSION**

Little is known about DNA repair and recombination in
rhizobia, and more generally in alpha-Proteobacteria.
Nevertheless, the fact that S. meliloti encodes multiple Ku proteins suggests that DSB repair could be important for its persistence in the environment [15]. In this study, we showed for the first time that RecA is involved in S. meliloti protection against several DNA damaging conditions, including NO, peroxynitrite, H$_2$O$_2$ and desiccation, while Ku proteins are involved in resistance against H$_2$O$_2$ and desiccation. RecA, apart from its role in HR, is also involved in the SOS response, controlling the expression of genes involved in other DNA repair pathways such as translesion synthesis and nucleotide excision repair [3]. Therefore, the sensitivity of recA mutant cells can be the consequence of a deficiency in DSB repair, due to HR inactivation, and/or result from the lack of SOS-induced repair mechanisms. On the other hand, Ku proteins play a role in DSB repair only. Thus, the involvement of Ku proteins in S. meliloti resistance against H$_2$O$_2$ and desiccation can be interpreted as a default in the repair of DSBs induced by these conditions.

The effect of Ku proteins on DSB repair was not visible in exponential phase and was only detected in stationary phase bacteria, whatever the stress considered. This is consistent with previous observations in other bacteria that HR is the main pathway of DSB repair in growing cells and that NHEJ becomes critical only in stationary phase [10, 11]. S. meliloti is present in various states of growth in nodules, including growing and non-growing bacteria in infection threads, growing young bacteroids just released in plant cells and non-growing fully differentiated bacteroids [34]. Therefore, testing whether RecA and Ku are involved in symbiosis was highly relevant.

Although H$_2$O$_2$, NO and peroxynitrite are encountered by S. meliloti during its symbiotic interaction with Medicago plants [35, 36], we found that the recA ku1234 mutant is not affected in its symbiotic efficiency with M. truncatula. Several hypotheses can be proposed to interpret this observation. First, S. meliloti encodes several catalases/peroxydases.

![Fig. 5. Peroxynitrite sensitivity of S. meliloti strains WT, Δku1234, ΔrecA and ΔrecA Δku1234. Sensitivity of strains was determined by addition of 1 mM peroxynitrite to S. meliloti cultures in exponential (a) and stationary (b) phases. Bacteria were plated after serial dilution. The figure shows a representative experiment from three independent biological experiments.](image)

Fig. 5. Peroxynitrite sensitivity of S. meliloti strains WT, Δku1234, ΔrecA and ΔrecA Δku1234. Sensitivity of strains was determined by addition of 1 mM peroxynitrite to S. meliloti cultures in exponential (a) and stationary (b) phases. Bacteria were plated after serial dilution. The figure shows a representative experiment from three independent biological experiments.

![Fig. 6. H$_2$O$_2$ sensitivity of S. meliloti strains WT, Δku1234, ΔrecA and ΔrecA Δku1234. (a) H$_2$O$_2$ sensitivity of strains was determined by disc diffusion assay. The means and standard deviations of the diameter of inhibition halos from three independent experiments are indicated for each strain. (b) H$_2$O$_2$ sensitivity of strains was determined by addition of 30 mM H$_2$O$_2$ for 30 min to exponential (light grey) and stationary (dark grey) phase S. meliloti cultures. The survival of each strain was determined by comparison with non-treated cells, and mutant survival was expressed relative to WT survival, taken as 100 %. The graph represents the mean of three independent biological experiments and error bars correspond to standard errors of the means. Statistical analyses were performed on log-transformed data using one-way ANOVA (P<0.01). The Bonferroni post-test was used to compare each mutant strain to the WT (*P<0.05; **P<0.01).](image)
RecA was also implicated in the desiccation resistance of Deinococcus radiodurans [32], Acinetobacter baumannii [44] and B. subtilis spores [45]. Mutants in the uvrABC genes, involved in nucleotide excision repair, were previously found to be more desiccation sensitive than the WT strain in S. meliloti [27]. Expression of uvrABC genes is under the control of the SOS response, which suggests that RecA involvement in desiccation resistance could be related to its role in the SOS response rather than in DSB repair. We therefore assume that desiccation primarily induces the formation of DNA lesions that are not DSBs. However, the finding that Ku proteins have a function in S. meliloti desiccation resistance, either in a recA background or in late desiccation stages (Fig. 7), conclusively demonstrates the formation of DSBs and the need for NHEJ to repair them. These DSBs could result from unrepaired lesions in the recA background or from progressive recA inactivation or appearance of higher constraints on DNA in the late desiccation stages. Ku was previously implicated in desiccation resistance in B. subtilis and Mycobacterium smegmatis [10, 46]. In Bradyrhizobium japonicum, nine genes whose products are involved in DNA repair were found among the genes up-regulated under desiccation conditions [47], and one of these encoded a putative Ku orthologue, suggesting that DSB repair is probably implicated also in the desiccation resistance of B. japonicum. Therefore, DSB repair could be important regarding the persistence of rhizobia in the soil.

In conclusion, this work highlights the importance of RecA and/or Ku proteins in repairing DNA damage, including DSBs, which may arise in S. meliloti genomic DNA under natural conditions. Despite this, DSB repair was found to be non-essential for the symbiosis of S. meliloti with M. truncatula, whereas it proved to play an important role in bacterial resistance to desiccation. This last observation could possibly be exploited in the production of new rhizobial strains with better properties as seed inocula in agriculture.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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